Nox1, Nox4 and Nox5, promote vascular permeability and neovascularization in retinopathy

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Abstract

Hypertension is a risk factor for the vascular permeability and neovascularization that threatens vision in diabetic retinopathy (DR). Excess reactive oxygen species derived from the NADPH oxidase (Nox) isoforms, Nox1 and Nox4, contributes to vasculopathy in DR, however, if Nox1/4 inhibition is beneficial in hypertensive DR is unknown. Here, we determined that diabetic spontaneously hypertensive rats (SHR) had exacerbated retinal vascular permeability and expression of angiogenic and inflammatory factors, compared to normotensive diabetic Wistar Kyoto rats (WKY). GKT136901, a specific dual inhibitor of Nox1 and Nox4, prevented these events in diabetic WKY and SHR. Retinal neovascularization does not develop in diabetic rodents and therefore the oxygen-induced retinopathy (OIR) model is used to evaluate this pathology. We previously demonstrated that Nox1/4 inhibition reduced retinal neovascularization in OIR. However, although Nox5 is expressed in human retina, its contribution to retinopathy has not been studied *in vivo*, largely due to its absence from the rodent genome. We generated transgenic mice with inducible human Nox5 expressed in endothelial cells (Ve-CAD^{+}Nox5^{+}mice). In Ve-CAD^{+}Nox5^{+} mice with OIR, retinal vascular permeability and neovascularization as well as the expression of angiogenic and inflammatory factors were increased compared to wild-type littermates. In bovine retinal endothelial cells which express Nox1, Nox4 and Nox5, Nox1/4 inhibition as well as Nox5 silencing RNA reduced the high glucose-induced up-regulation of oxidative stress, angiogenic and inflammatory factors. Collectively, these data indicate the potential of Nox1, Nox4 and Nox5 inhibition to reduce vision-threatening damage to the retinal vasculature.

Introduction

Diabetic retinopathy (DR) is a leading cause of vision loss and blindness across the globe with approximately 93 million people estimated to have DR and 29 million people vision-threatening DR.[1] The clinical hallmark of DR is damage to the retinal vasculature characterized by vascular permeability and oedema due to breakdown of the blood-retinal barrier (BRB) as well as neovascularization.[2] This vascular pathology is driven by a number of factors including vascular permeability and angiogenic agents such as vascular endothelial growth factor (VEGF), and pro-inflammatory molecules,[2] as well as hypertension which is a risk factor for DR[3, 4] and can damage the retina in the absence of diabetes.[5] Agents to inhibit VEGF have revolutionised the treatment of DR,[6] and anti-hypertensive therapies such as blockers of the angiotensin type 1 receptor have beneficial effects.[5, 7] However, these approaches are not completely retinoprotective[7] which has led to considerable interest in targeting other factors that have a causal role in DR.

Oxidative stress, the excess production of reactive oxygen species (ROS), is a major contributor to diabetic complications including DR.[8] Excess ROS promotes the production of angiogenic and inflammatory factors[9, 10] that cause the increased vasculopathy and inflammation that develops in DR.[11, 12] NADPH oxidase (Nox) is of interest as a treatment target for DR as this enzyme family's sole function is the production of ROS.[13] There are seven known Nox isoforms of which Nox1, Nox2 and Nox4 are implicated in the pathogenesis of vascular dysfunction and inflammation in various retinal diseases.[14, 15] Of growing interest is the role of Nox5 in vascular disease as it is the only calcium-activated Nox isoform present in endothelium and it promotes endothelial cell proliferation[16] and hypertension.[17] Nox5 is expressed in human retina,[15] but is not easily studied *in vivo* due to its absence from the mouse and rat genome, albeit Nox5 is expressed in other species such as cows and rabbits.[18]

Multiple pathways are involved in the increase in Nox and ROS levels that occur in hypertension and diabetes including the renin-angiotensin system, sheer stress on blood vessels, the hyperglycaemic-induction of metabolic pathways and inflammation.[10 19, 20 21] However, treatment strategies to block the excess production of ROS derived from Nox have until recently been hindered by the absence of agents that inhibit specific Nox isoforms.[13] GKT136901 and GKT137831 are two structurally related compounds that exhibit potent dual action inhibition of Nox1 and Nox4 and have beneficial effects in various pre-clinical models of disease.[15, 22-24] We hypothesized that GKT136901 would have retinoprotective effects in hypertensive DR and therefore studied spontaneously hypertensive rats (SHR) with streptozotocin diabetes and made comparisons to normotensive Wistar Kyoto rats (WKY). Further, we reasoned that Nox5 would promote retinal neovascularization and thus studied endothelial specific Nox5 transgenic mice in the oxygen-induced retinopathy (OIR) model as well as primary cultures of bovine retinal endothelial cells (BREC) since cows express Nox5.

Methods

All methods are available within the article and online-only Data Supplement. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Body weight, blood glucose and blood pressure

The results are summarised in Table S1 (online-only Data Supplement). Diabetes reduced body weight and increased blood glucose levels in WKY and SHR but had no effect on systolic blood pressure. GKT136901 had no effect on body weight, blood glucose and systolic blood pressure. Ve-Cad^{+}Nox5^{+} mice had similar body weights (6.4±0.2g) as their

littermate controls (VE-Cad^{-}Nox5^{+}, 6.5±0.1g; VE-Cad^{+}Nox5^{-}, 6.9±0.1g; VE-Cad^{+}Nox5^{+}, 6.8±0.2g).

Nox expression in retina of hypertensive and diabetic rats

The mRNA levels of Nox1, Nox2 and Nox4 in retina were increased in non-diabetic SHR compared to non-diabetic WKY (Figures 1A to 1C). Nox1, Nox2 and Nox4 mRNA were increased in diabetic WKY compared to non-diabetic WKY controls. In SHR, only Nox4 mRNA levels were increased by diabetes compared to non-diabetic SHR (Figure 1C).

Retinal oxidative stress is prevented by Nox1/4 inhibition in rats

To evaluate oxidative stress, two complementary approaches were used. Firstly, immunohistochemistry was performed for 8-hydroxyguanine (8-OHdG) a major product of DNA oxidation and secondly, total superoxide levels were measured with the lucigenin assay. 8-OHdG immunolabeling was present at low levels in ganglion cells in non-diabetic WKY (Figures 1D and 1E). Hypertension augmented 8-OHdG immunolabeling in ganglion cells and it was also present in the inner nuclear layer (Figures 1D and 1E). Diabetes in WKY and SHR increased 8-OHdG in these sites. These findings are consistent with reports of increased ROS levels in ganglion cells which may contribute to neurodegeneration.[25] Immunolabeling was also present in the cell processes of macroglial Müller cells (Figures 1D and 1E). In diabetic WKY and SHR, GKT136901 reduced 8-OHdG immunolabeling to almost the level of respective non-diabetic controls (Figures 1D and 1E). Superoxide levels in retina were not increased in non-diabetic SHR but elevated in diabetic WKY and SHR and reduced to control levels with GKT136901 treatment (Figure 1F).

Retinal vascular permeability induced by diabetes is exacerbated in SHR and prevented by Nox1/4 inhibition

Vascular permeability in DR is influenced by growth factors which promote impaired endothelial barrier function.[26] Vascular leakage into the retina and vitreous cavity were increased by diabetes in WKY and further increased in SHR, with vascular leakage highest in diabetic SHR (Figures 2A and 2B). Nox1/4 inhibition prevented vascular leakage into both the retina and vitreous of WKY and SHR with diabetes (Figures 2A and 2B).

VEGF and angiopoietin-2 are key retinal vascular permeability and angiogenic factors in DR.[26] Diabetic WKY exhibited elevated levels of VEGF and angiopoietin-2 in retina as well as VEGF in vitreous compared to non-diabetic WKY (Figures 2C to 2F). In retina, VEGF mRNA and protein levels were further increased in diabetic SHR (Figures 2C and 2E); however, angiopoietin-2 mRNA levels were similar in WKY and SHR with diabetes (Figure 2F). In diabetic WKY and SHR, GKT136901 prevented the increase in VEGF and angiopoietin-2 with levels similar to their respective non-diabetic controls (Figures 2C to 2F).

An important component of impaired endothelial barrier function is the decreased expression of endothelial cell tight junctional proteins, zona occludin-1 (ZO-1) and occludin.[27] Diabetes reduced the expression of ZO-1 and occludin in retina compared to non-diabetic WKY (Figure S1, on-line Data Supplement). In retina of SHR, these junctional proteins were reduced compared to normotensive non-diabetic WKY, and further reduced with diabetes. Nox1/4 inhibition prevented the decrease in ZO-1 and occludin in the retinas of WKY and SHR with diabetes, although the protection afforded by GKT136901 was most striking in diabetic WKY for occludin (Figure S1, on-line Data Supplement).

Diabetes-induced Müller cell gliosis and inflammation were increased in SHR and reduced by Nox1/4 inhibition

Reactive gliosis is indicative of BRB breakdown and features increased levels of glial fibrillary acidic protein (GFAP) in Müller cells and astrocytes.[27] Immunolabeling for GFAP was minimal and largely on the retinal surface in non-diabetic WKY but increased and present in Müller cell and astrocyte processes in retinas from diabetic WKY (Figure 3A). GFAP immunolabeling was increased in non-diabetic SHR compared to non-diabetic WKY, with the highest expression in diabetic SHR. Nox1/4 inhibition reduced GFAP in WKY and SHR with diabetes to the level of WKY non-diabetic controls (Figure 3B).

Inflammatory factors contribute to the development of DR.[28] The mRNA levels of intracellular adhesion molecule-1 (ICAM-1) and tumour necrosis factor-α (TNFα) in the retina were increased in non-diabetic SHR compared to non-diabetic WKY (Figures 3C and 3D). Diabetes increased these inflammatory factors in the retinas of both rat strains with the highest levels in diabetic SHR. GKT136901 prevented the diabetes-induced increase ICAM-1 and TNFα in retinas of WKY and SHR (Figures 3C and 3D). The protein levels of monocyte chemotactic protein-1 (MCP-1) in retina and vitreous were increased by diabetes in both WKY and SHR (Figures 3E and 3F) and further increased by hypertension in diabetic SHR (Figure 3E). GKT136901 reduced MCP-1 protein levels in retina and vitreous of SHR and WKY with diabetes (Figures 3E and 3F).

Nox1/4 inhibition reduced damage to retinal endothelial cells *in vitro*

To further examine the effect of Nox1/4 inhibition on the retinal vasculature we studied primary cultures of BREC. The expression of Nox1 and Nox4, but not Nox2 were increased by 72 hours of high glucose (Figure 4A). As cows like humans express Nox5, we evaluated this Nox isoform and found increased mRNA levels following exposure to high glucose (Figure 4A). In BREC exposed to high glucose, ROS levels measured by dihydroethidium (DHE) flow cytometry, were increased compared to normal glucose controls, and reduced

with GKT136901 (Figure 4B). High glucose reduced ZO-1 protein levels, which were restored by GKT136901 (Figures 4C and 4D). ICAM-1, a marker of vascular inflammation, was increased by high glucose and reduced by GKT136901 (Figure 4E).

Nox5 inhibition reduced high glucose-induced angiogenic and inflammatory factors in retinal endothelial cells *in vitro*.

The increased expression of Nox5 in BREC following exposure to high glucose led us to investigate the effect of silencing Nox5 using small interfering RNAs (si-Nox5). In BREC, si-Nox5 reduced Nox5 protein levels by 40% and mRNA levels by 60% (Figure S2, online-only Data Supplement), while the expression of Nox1, Nox2 and Nox4 were not affected by si-Nox5 compared to BREC treated with a scrambled siRNA (si-scr) (Figure S3, online-only Data Supplement). As GKT136901 inhibits Nox5, although with less efficiency than Nox1 and Nox4,[13] we evaluated if this compound influenced Nox5 expression in BREC. GKT136901 reduced the high glucose-induced increase in Nox5 mRNA (Figure S4, on-line Data Supplement). Next, we determined that ROS levels measured by DHE flow cytometry were increased in BREC exposed to high glucose and reduced with si-Nox5 (Figures 5A and 5B). Furthermore, VEGF mRNA and protein levels (Figures 5C and 5D) as well as ICAM-1 mRNA levels (Figure 5E) were elevated in BREC exposed to high glucose and reduced with si-Nox5.

Nox5 exacerbated retinal oxidative stress and neovascularization in OIR

With *in vitro* studies clearly demonstrating a key role for Nox5 in promoting oxidative stress and modulating glucose-induced expression of angiogenic and inflammatory factors the ability of Nox5 to influence retinal vascular disease *in vivo* was explored. Endothelial cell-specific Nox5 transgenic (VE-Cad^{+}Nox5^{+}) mice were subjected to OIR, a model of retinal

neovascularization. We first confirmed Nox5 expression in these transgenic mice by real-time PCR. Nox5 mRNA was detected in the retinas of VE-Cad^{+}Nox5^{+} mice (Ct numbers 31-32, Figure S5A, online-only Data Supplement) while their littermate controls had undetectable levels of Nox5 mRNA as predicted since Nox5 is not present in the mouse genome. The ability of Nox5 to induce oxidative stress was confirmed by increased superoxide levels in the retinas of VE-Cad^{+}Nox5^{+}mice, while the levels of other Nox isoforms were unchanged compared to littermate controls (Figures S5B to S5E, online-only Data Supplement). Next, we examined if VE-Cad^{+}Nox5^{+} mice had increased retinal vasculopathy. VE-Cad^{+}Nox5^{+} mice with OIR had enhanced retinal neovascularization (Figures 6A and 6B) and vascular leakage (Figure 6C) compared to OIR controls. Furthermore, VEGF protein and mRNA levels were elevated (Figures 6D and 6E) as well as ICAM-1 levels (Figure 6F) in retina compared to OIR controls.

Discussion

To our knowledge this is the first study to identify that a specific dual inhibitor of Nox1/4 prevents the vision-threatening damage to the retinal vasculature that occurs due to diabetes in the setting of concomitant hypertension. This is important since most subjects with vision threatening DR have systemic hypertension. In both normotensive WKY and hypertensive SHR with diabetes, the Nox inhibitor GKT136901 protected the BRB by reducing vascular permeability as well as VEGF levels within the retina and vitreous. These vasculo-protective actions were confirmed *in vitro* and *in vivo*, with GKT136901 preventing the diabetes-induced decline in the endothelial cell tight junctional proteins, ZO-1 and occludin, which are critical for BRB integrity. Our findings are likely to be relevant to patients with hypertension and DR as both Nox1 and Nox4 are present in human retina.[15] Nevertheless, we suggest that a potential causal role for Nox5 in retinal vasculopathy cannot be overlooked due to the

expression of this Nox isoform in human retina,[15] and the ability of Nox5 to promote angiogenic responses in endothelial cells *in vitro.*[16] Our *in vivo* and *in vitro* data strongly support this postulate as we present new evidence that overexpression of Nox5 in the endothelium exacerbates retinal neovascularization and vascular permeability. Furthermore, Nox5 siRNA ameliorates high glucose-mediated damage to retinal endothelial cells.

Diabetes and hypertension can compromise the integrity of the BRB resulting in vision-threatening vascular leakage,[29] pathology linked to oxidative stress.[12, 25, 30] Indeed, ROS levels increase in the rat retina a few weeks after the onset of diabetes preceding the development of retinal vasculopathy.[31, 32] Our findings are in agreement since 8-OHdG immunolabeling increased in ganglion cells and macroglial Müller cells of SHR retina. Diabetes is a potent inducer of oxidative stress as evidenced by increased 8-OHdG immunolabeling and superoxide levels in WKY and SHR retina. 8-OHdG was further increased in diabetic SHR compared to diabetic WKY suggesting that hypertension has an additive effect on retinal oxidative damage in diabetes. These findings together with the elevated levels of Nox1, Nox2 and Nox4 transcripts in SHR retina indicate that Nox1/4 inhibition might attenuate oxidative stress and retinal vascular pathology in diabetes. A limitation of our study is that we did not measure ROS and the protein levels of Nox isoforms early in diabetes and hypertension.[31, 32] Nevertheless, our data that Nox1/4 inhibition markedly reduces oxidative stress in the retina, emphasise the potential of this treatment approach to attenuate the excess production of ROS that occurs in the retina in diabetes and hypertension.

The BRB in the inner retina regulates the transport of fluids and proteins across endothelial cells, with tight junctional complexes having a key role in maintaining the integrity of the barrier.[33] Further, macroglial Müller cells, whose processes are closely associated with the BRB, induce barrier properties in retinal endothelial cells.[34, 35] Breakdown

of the BRB is highly influenced by VEGF produced by Müller cells which down-regulates the expression of tight junctional proteins such as occludin.[36, 37] Our findings are consistent with our previous studies in diabetic rats demonstrating that retinal Müller cell gliosis and VEGF levels are increased as well as vascular leakage into the retina and vitreous, and that these events are exacerbated by systemic hypertension.[38] We also identified that angiopoietin-2 levels are increased in retinas of diabetic WKY and SHR, which is of interest due to the ability of this factor to potentiate the actions of VEGF.[39] Another indication that the integrity of the BRB was compromised in diabetic WKY and SHR was the reduced expression levels of ZO-1 and occludin which were further lowered by the combination of hypertension and diabetes.[27] Importantly, Nox1/4 inhibition prevents the aforementioned damage to the retinal vasculature. These findings were confirmed in primary cultures of BREC, where the high glucose-induced increase in ROS and reduction in ZO-1 was ameliorated by GKT136901. Of interest is that Nox1/4 inhibition improved retinal pathology in SHR in the presence of elevated blood pressure. These data suggest that reducing excess ROS in the retina is a more important driver of diabetic and hypertensive retinopathy than lowering blood pressure. These findings might explain why angiotensin type 1 receptor blockade is only partially retinoprotective in patients with hypertension and DR.[7, 8] Nevertheless, we acknowledge that in our study, blood pressure in rats was not measured using radio-telemetry, a technique that could potentially reveal reductions in blood pressure following treatment with the Nox1/4 inhibitor.

Evidence that Nox1, Nox2 and Nox4 isoforms have a causal role in various disorders has continued to expand, but far less understood is the role of Nox5 in disease including retinopathy.[18] Since Nox5 is absent from the rodent genome, mouse models expressing the human Nox5 gene have been developed.[40-42] In transgenic mice expressing Nox5 under the control of the Tie2 promoter, disruption of the blood-brain barrier due to stroke was increased

in transgenic Nox5 mice, indicating a critical role for Nox5 in vascular integrity in the central nervous system.[40] Our findings in OIR are in agreement, with Nox5 overexpression in the vasculature, exacerbating breakdown of the BRB as reflected by increased vascular leakage and VEGF expression. Neovascularization within the inner retina and into the vitreous is a hallmark feature of end-stage proliferative DR,[2] however, this vascular pathology does not develop in diabetic rodents. OIR re-capitulates the neovascularization that occurs in retinopathy of prematurity in children,[43] and has similarities to proliferative DR. It has been reported that knockdown of Nox4 by adenovirus delivered siRNA reduced retinal neovascularization in OIR,[44] and Nox1/4 inhibition attenuated retinal neovascularization and VEGF levels in this model.[15] We present novel *in vivo* evidence that overexpression of Nox5 in endothelial cells exacerbates retinal neovascularization in OIR. These data support previous *in vitro* studies showing that overexpression of Nox5 enhances endothelial cell proliferation and tubule formation.[16]

To evaluate the therapeutic potential of our findings we utilized BREC which we reported express Nox5 as well as Nox1, Nox2 and Nox4.[15] Here, we identified that the mRNA levels of these Nox isoforms except for Nox2 are increased by high glucose in BREC. These findings are somewhat different from our data in rats where Nox2 mRNA levels in whole retina were increased with diabetes. The reasons for these differences are unclear but might indicate that Nox expression levels in whole retina do not necessarily reflect expression levels in individual cell populations such as endothelial cells. The ability of Nox5 siRNA as well as Nox1/4 inhibition to attenuate the high glucose-induced up-regulation of ROS and VEGF in BREC highlights the vasculo-protective effects of reducing Nox5 as well as Nox1 and Nox4 in diabetes. A limitation of our study was that Nox5 siRNA did not completely reduce Nox5 mRNA and protein levels, with a greater suppression of Nox5 potentially eliciting further reductions in ROS.

Inflammation contributes to endothelial cell injury in the retina with ICAM-1 influencing leukocyte adhesion.[28] Our data emphasize the capacity of Nox1/4 inhibition to attenuate the up-regulation of ICAM-1 in the retina caused by diabetes and exacerbated by hypertension. We determined that the high glucose-mediated increase in endothelial cell derived ICAM-1 is particularly responsive to Nox1/4 inhibition as well as to down-regulation of Nox5. These findings highlight the importance of reducing the expression of certain Nox isoforms in DR. The inflammatory mediators, TNFα and MCP-1, are produced by various cell types within the retina[45, 46] and influence the endothelial cell dysfunction that occurs in response to diabetes and hypertension. Indeed, these factors not only stimulate the adherence of leukocytes to the vasculature, but also facilitate breakdown of the BRB in DR.[28, 47] The ability of Nox1/4 inhibition to attenuate the up-regulation of TNFα and MCP-1 in the retina of diabetic WKY and SHR adds support to evidence that Nox1/4 inhibition has potent anti-inflammatory actions in retinopathy[48] and other settings such as diabetes-associated atherosclerosis and nephropathy.[23, 49, 50]

Perspectives

Targeting Nox isoforms is a potential treatment for DR. We demonstrated that Nox1/4 inhibition protects the BRB in diabetes and in the common clinical scenario when hypertension is superimposed. The role of Nox5 in retinopathy has been difficult to study due to its absence from the rodent genome. We provide new evidence that Nox5 is involved in retinal vasculopathy. Collectively, these findings provide a rationale for investigating Nox5 inhibitors alongside Nox1/4 inhibition as a new treatment approach for DR.

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Conflicts of Interest/Disclosures

None.

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Novelty and Significance

What is New

- Nox1/4 inhibition protects the blood-retinal barrier and reduces oxidative stress and inflammation induced by diabetes and hypertension.
- Nox5 promotes retinal neovascularization and vascular permeability.

What is Relevant

Inhibition of Nox1/4 as well as Nox5 may offer a unique approach to preventing damage to the retinal microvasculature.

Summary

Oxidative stress threatens the BRB, with Nox1, Nox4 and Nox5 having a causal role. Strategies to inhibit these Nox isoforms may be more efficacious than targeting single Nox isoforms and thereby prevent the progression to vision loss in diabetes and hypertension.

Figure 1. Oxidative stress in DR is exacerbated in SHR and reduced by Nox1/4 inhibition.

NDB, non-diabetic. DB, diabetic. GKT, GKT136901. GCL, ganglion cell layer. IPL, inner plexiform layer. INL, inner nuclear layer. ONL, outer nuclear layer. RLU, relative luminescence unit. (**A**) Nox1, (**B**) Nox2 and (**C**) Nox4 mRNA levels in retina. *n*=5-9 rats per group. (**D**) Representative images showing 8-OHdG immunolabeling in 3μm paraffin sections. Counterstain, haematoxylin. 8-OHdG in non-diabetic WKY is minimal. 8-OHdG in the GCL (arrows) and INL is increased in non-diabetic SHR, and further increased in diabetic WKY and SHR in these locations as well as Müller cell processes (arrowheads). GKT136901 reduced 8-OHdG immunolabeling in diabetic rats. Scale bar, 40μm. (**E**) 8-OHdG quantitation. *n*=5-6 rats per group. (**F**) Superoxide levels in retina are unchanged with hypertension but increased with diabetes in WKY and SHR. GKT136901 reduced superoxide levels in retina of diabetic rats. *n*=4-6 rats per group. **P*<0.05, ***P*<0.01, ****P*<0.001. Mean±SD.

Figure 2. Diabetes-induced retinal vascular permeability is exacerbated in SHR and reduced by Nox1/4 inhibition.

NDB, non-diabetic. DB, diabetic. GKT, GKT136901. Vascular leakage measured by ELISA for albumin in (**A**) retina and (**B**) vitreous. *n*=5-8 rats per group. VEGF (**C**) mRNA and (**D**) protein levels in retina. (**E**) VEGF protein levels in vitreous and (**F**) angiopoietin-2 (Ang2) mRNA levels in retina. *n*=5-9 rats per group. **P*<0.05, ***P*<0.01, ****P*<0.001. Mean±SD.

Figure 3. Diabetes-induced retinal gliosis and inflammation are exacerbated in SHR and reduced by Nox1/4 inhibition.

NDB, non-diabetic. DB, diabetic. GKT, GKT136901. ILM, inner limiting membrane. GCL, ganglion cell layer. IPL, inner plexiform layer. INL, inner nuclear layer. ONL, outer nuclear layer. (**A**) Representative images showing GFAP immunolabeling in 3μm paraffin sections. GFAP in non-diabetic WKY is restricted to the retinal surface at the ILM (asterisk) adjacent to the vitreous. GFAP is present in Müller cell processes (arrowheads) in non-diabetic SHR and increased with diabetes in WKY and SHR. GKT136901 reduced GFAP in diabetic rats. Scale bar, 40μm. (**B**) GFAP quantitation. *n*=5 rats per group. (**C**) ICAM-1 and (**D**) TNFα mRNA levels in retina. MCP-1 protein levels in retina (**E**) and vitreous (**F**). *n*=5-9 rats per group. **P*<0.05, ***P*<0.01, ****P*<0.001. Mean±SD.

Figure 4. Nox1/4 inhibition reduced high glucose-induced damage to retinal endothelial cells *in vitro.*

NG, normal glucose. HG, high glucose. GKT, GKT136901. Experiments were performed in primary cultures of bovine retinal endothelial cells. (**A**) Nox isoform mRNA levels. (**B**) Mean fluorescent intensity (MFI) for dihydroethidium (DHE) to detect ROS by flow cytometry. (**C**) Western blots for ZO-1. (**D**) VEGF and (**E**) ICAM-1 mRNA levels. *n*=6-12 samples from 2 to 3 independent experiments. **P*<0.05, ***P*<0.01 and ****P*<0.001. Mean±SD.

Figure 5. Nox5 inhibition reduced high glucose-induced angiogenic factors and inflammation in retinal endothelial cells *in vitro*.

NG, normal glucose. HG, high glucose. Experiments were performed in primary cultures of bovine retinal endothelial cells. (**A**) Mean fluorescent intensity (MFI) for dihydroethidium (DHE) to detect ROS by flow cytometry. (**B**) si-Nox5 reduced high glucose-induced ROS levels. (**C**) VEGF protein levels in supernatants. (**D**) VEGF and (**E**) ICAM-1 mRNA levels.

*P<0.05, **P<0.01, ***P<0.001. n=9-12 samples from 3 independent experiments. Mean±SD.

Figure 6. Nox5 exacerbates retinal neovascularization and inflammation in OIR.

NV, neovascularization. Relative luminescence unit, RLU. **(A)** Representative flat-mounts of retinas from mice with oxygen-induced retinopathy (OIR) stained with FITC-conjugated isolectin B4 to identify the vasculature at postnatal day 18. Upper panel shows the entire retina. Yellow boxes in the upper panel are shown in higher magnification in the lower panel. Arrowheads show areas of NV. Scale bar=0.125mm. (**B**) Transgenic mice expressing Nox5 in endothelial cells (Ve-Cad^{+}Nox5^{+}) developed more extensive retinal NV than OIR littermate controls. (**C**) Retinal vascular leakage measured by albumin ELISA. (**D**) VEGF protein, (**E**) VEGF mRNA and (**F**) ICAM-1 mRNA levels are increased in retinas of Ve-Cad^{+}Nox5^{+} OIR mice. *P<0.05, **P<0.01, ***P<0.001. n=6-9 mice per group. Mean±SD.